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Superantigen-induced cytokine release from whole-blood cell culture as a functional measure of drug efficacy after oral dosing in nonhuman primates

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Abstract

Evaluation of drug efficacy for human diseases is routinely performed in animal models for efficiency and in accordance with FDA regulations. Rhesus macaques have been used as models for various lethal diseases and correlates of immunity, as nonhuman primates (NHP) closely resemble humans. We examined the ex vivo cytokine response of superantigen-stimulated whole-blood cells as a first step to therapeutic efficacy testing for bacterial superantigen-induced shock in NHP after oral dosing of pentoxifylline. Doses of 120 mg/kg of pentoxifylline effectively attenuated staphylococcal enterotoxin B-induced tumor necrosis factor α (TNFα), gamma interferon (IFNγ) and interleukin 2 (IL-2) in ex vivo culture of NHP whole-blood cells by 88%, 81%, and 76%, respectively, whereas lower doses of 48 or 72 mg/kg had no inhibitory effect. Thus cytokine release of stimulated peripheral blood cells provides a convenient biological measurement of the anti-inflammatory potency of pentoxifylline and has the advantage of assessing functional responses to a specific biotoxin of interest.
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Keywords: Pentoxifylline; Cytokines; Whole-blood cell culture

1. Introduction

Staphylococcal enterotoxin B (SEB) and related superantigenic toxins are potent activators of the immune system and cause a variety of human diseases, ranging from food poisoning to toxic shock (Kotzin et al., 1993; Fraser et al., 2000; McCormick et al., 2001; Baker and Acharya, 2003; Proft and Fraser, 2003). These toxins bind to both MHC class II molecules and specific Vβ regions of T cell receptors (Choi et al., 1989; Mollick et al., 1991), resulting in the activation of both monocytes/macrophages and T lymphocytes (Krakauer, 1999; Proft and Fraser, 2003). The interactions of these toxins with host cells lead to excessive production of proinflammatory cytokines and T cell proliferation, causing clinical symptoms that include fever, hypotension, and shock (Kotzin et al., 1993; Fraser et al., 2000).

At present, there is no known treatment for staphylococcal exotoxins (SE)-induced shock except for the use of intravenous immunoglobulins (Darenberg et al., 2004). Various strategies have been devised to treat SE-induced diseases including blocking SE binding to host cells via interaction with MHC class II molecules or T cell receptor (Krakauer, 1999; Visvanathan et al., 2001; Geller-Hong and Gupta, 2003; Rajagopalan et al., 2004). We and others previously showed that induction of the proinflammatory cytokines and the signaling pathways used by SE are important targets as excessive proinflammatory cytokines mediate the toxic effects of SE (Parsonnet, 1989; Trede et al., 1991; Miethke et al., 1993; Stiles et al., 1993;

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14. ABSTRACT

Evaluation of drug efficacy for human diseases is routinely performed in animal models for efficiency and in accordance with FDA regulations. Rhesus macaques have been used as models for various lethal diseases and correlates of immunity, as nonhuman primates (NHP) closely resemble humans. We examined the ex vivo cytokine response of superantigen-stimulated whole-blood cells as a first step to therapeutic efficacy testing for bacterial superantigen-induced shock in NHP after oral dosing of pentoxifylline. Doses of 120mg/kg of pentoxifylline effectively attenuated staphylococcal enterotoxin B-induced tumor necrosis factor alpha (TNFalpha), gamma interferon (IFNgamma) and interleukin 2 (IL-2) in ex vivo culture of NHP whole-blood cells by 88%, 81%, and 76%, respectively, whereas lower doses of 48 or 72mg/kg had no inhibitory effect. Thus cytokine release of stimulated peripheral blood cells provides a convenient biological measurement of the anti-inflammatory potency of pentoxifylline and has the advantage of assessing functional responses to a specific biotoxin of interest.

15. SUBJECT TERMS
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Krakauer, 2005). TNFα and IFNγ are key mediators in SEB-induced toxic shock, and in vivo studies also show a correlation between increased serum levels of these cytokines with SEB-induced lethality (Miethke et al., 1992; Stiles et al., 1993). Genetic knockout mice deficient in TNF receptor type 1 (TNFR1) or IFNγ receptor are also resistant to SEB-induced shock (Stiles et al., 1999). Neutralizing antibodies against TNFα prevented SEB-induced lethality (Miethke et al., 1992). Murine models are used most often in therapeutic efficacy studies because immunological reagents are available and testing can be done quickly and economically, especially when lethality is used as an endpoint (Krakauer and Stiles, 2006). However, species differences indicate mouse models often do not mimic human diseases and nonhuman primates (NHP) are often used as human surrogates. In particular, in vivo studies with SE using mouse models require potentiating agents to enhance the toxic effects of SE (Stiles et al., 1993; Blank et al., 1997) because mice are less sensitive to SE due to lower affinity of SE to mouse MHC class II molecules (Mollick et al., 1991). Rhesus macaques have been used as a lethal model for inhaled SEB, as pathogenic features of SEB intoxicated monkeys resemble those of humans (Hodoval et al., 1968). Therefore, NHP may provide a consistent model for the development of therapeutics for SE-induced shock in humans. It is not known at present if drugs effective in mouse models of SEB intoxication can be applied to NHP or human.

Pentoxifylline is a methylxanthine derivative that inhibits the production of TNFα by endotoxin- or SEB-stimulated human peripheral blood mononuclear cells (Neuner et al., 1994; Krakauer and Stiles, 1999) and is effective in reducing serum cytokines in mice with SEB-mediated shock (Krakauer and Stiles, 1999). The present study was undertaken to provide a first estimate for a therapeutic dose of pentoxifylline in attenuating superantigen-mediated inflammatory mediators ex vivo in NHP after oral dosing. Additionally, we wanted to establish ex vivo culture conditions for NHP whole-blood cells as NHP represent a realistic animal model for therapeutic development against various human diseases.

2. Materials and methods

2.1. Reagents

SEB and SEA were obtained from Toxin Technology (Sarasota, FL). The endotoxin content of these SEB and SEA preparations was <1 ng of endotoxin per mg of SEB, as determined by the Limulus amoebocyte lysate gelation test (Biowhittaker, Frederick, MD). Pentoxifylline and other common reagents were obtained from Sigma (St. Louis, MO).

2.2. Animals

Twenty-four rhesus monkeys (*Macaca mulatta*), each weighing 4–8 kg, prescreened by an enzyme-linked immunosorbent assay to be free of antibodies to SEB were used in this study. The absence of anti-SEB antibodies in these monkeys provide assurance that results from this study would not be due to antibody neutralization or other interference. Monkeys were maintained in individual cages with full access to filtered tap water, and were fed commercial monkey chow, supplemented with fresh fruits. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations. All experiments involving animals adhered to principles stated in the Guide for Care and Use of Laboratory Animals, National Research Council, 1996. All animal manipulations were performed under an anesthetic dose (3–6 mg/kg intramuscularly) of Telazol® (tiletamine hydrochloride and zolazepam hydrochloride).

2.3. Pentoxifylline doses and regimens

Healthy NHP ($n = 4$) received pentoxifylline (starting at 48 mg/kg every 8 h) via orogastric tube every 8 h. Pentoxifylline was supplied as a 50 mg/ml aqueous solution. Sham-treated control animals ($n = 2$) received water orogastricly following exactly the same schedule as drug-treated NHP. The dose of pentoxifylline was increased in a series of three subsequent experiments to 72 mg/kg, 96 mg/kg and 120 mg/kg. Some NHP were reused after a resting period of at least 3 months. Blood was taken from monkeys immediately before the start of drug treatment (time = 0) and peripheral blood cells from each NHP were established as a baseline for that particular animal. Blood was collected again 3 h after the first dose of pentoxifylline and at 27 h after the first dose, i.e., 3 h after the fourth dose from each NHP. At every time point, blood cells were cultured within 30 min after collection. No side effects were observed in NHP receiving multiple doses of pentoxifylline.

2.4. Ex vivo whole-blood cell culture and cytokine assays

Blood was collected in EDTA tubes and diluted 1/5 with RPMI supplemented with 10% heat-inactivated fetal calf serum and plated in 24-well cell culture plates. Cells were stimulated with a predefined optimal concentration of 750 ng/ml of SEB or SEA for 20 h at 37°C. The concentration of superantigens is consistent with those previously used in in vitro studies using human peripheral blood mononuclear cells (Fraser et al., 1992; Krakauer and Stiles, 1999). Supernatants were harvested and kept frozen until assayed. Levels of TNFα, IFNγ and IL-2 present in supernatants were determined by ELISA kits, according to manufacturer's specifications (Biosource, Camarillo, CA).

2.5. Statistical analysis

Cytokine data from individual NHP are expressed as the mean pg/ml ± SE of duplicate samples. Inhibition of SE-stimulated cytokines by pentoxifylline is expressed as a percentage of SE-treated whole-blood cells obtained at zero

time before drug or sham treatment. Inhibition data were subsequently analyzed for significant differences by Student's *t*-test with Stata (Stata Corp., College Station, TX). The results were considered significant if P was <0.05.

3. Results

Pilot experiments were performed to determine cell culture conditions for NHP blood cells ex vivo. Blood cells obtained from normal NHP were stimulated with various concentrations of SEB to define the optimal dose of superantigen and culture time. Fig. 1a shows the variation of cytokine responses from individual monkeys to an optimal concentration of SEB (750 ng/ml). Similar results were observed with another superantigen SEA (Fig. 1b). The difference in cytokine levels among NHP was also observed with human peripheral blood mononuclear cells from different donors stimulated with superantigen (Krakauer, 1995). Differential binding affinities of SEB and other superantigens to different MHC alleles contribute to the heterogeneous response to these superantigens (Mollick et al., 1991; reviewed in Krakauer, 1999). Importantly, differences among the cytokines TNFα, IFNγ and IL-2 from each monkey were also evident. We chose these three cytokines released by superantigen-activated cells as biomarkers of activation because they are responsible for the pathogenic effects of SE (Miethke et al., 1993; Stiles et al., 1993). The cell sources for these three prototypical cytokines examined also differed. TNFα is produced mostly by activated monocytes and T cells, whereas T cells and NK cells are mainly IFNγ-producing cells. Moreover, these two cytokines act synergistically and excessive levels contribute to various inflammatory diseases (Krakauer et al., 1998). IL-2, produced by activated T cells in response to superantigens, is used often to assess T cell stimulation (Fraser et al., 1992). Due to the individual variations of cytokine levels among NHP, data are expressed as a percentage of cytokine release from SE-stimulated blood cells at time zero before initiation of drug dosing.

We next determined the dose of pentoxifylline necessary to achieve inhibition of cytokine release from SEB-stimulated blood cells ex vivo. A specific dose of pentoxifylline was administered orally every 8 h and peripheral blood was taken at 3 h and 27 h. Whole-blood cells were stimulated with SEB or SEA ex vivo for 20 h. The cytokine levels from whole-blood cells taken at 3 h or 27 h were compared to cytokines induced by SEB-stimulated cells at zero time before initiation of drug treatment (100%). NHP given 48 mg/kg and 72 mg/kg of pentoxifylline did not produce inhibitory effects at 3 h (data not shown). Fig. 2 indicates

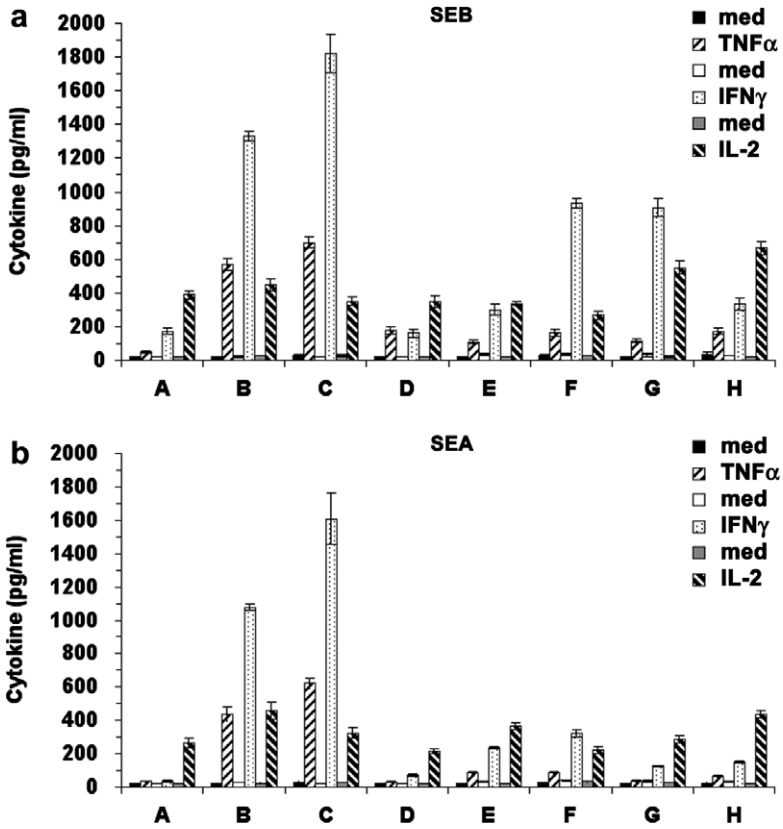

Fig. 1. Tumor necrosis factor α (TNFα), interferon gamma (IFNγ) and interleukin-2 (IL-2) production by (a) SEB and (b) SEA- stimulated whole-blood cells from normal NHP. Individual NHP are represented by A to H. Supernatants were harvested at 20 h for determination of cytokines by ELISA. Background levels of each cytokine from cells cultured in medium alone (med) are as indicated. Data are the mean pg/ml ± SE of duplicate samples and results represent eight NHP.

that 48 mg/kg and 72 mg/kg did not have suppressive effects at 27 h, whereas attenuation of IFNγ and IL-2 to 70% and 33%, respectively, was observed in ex vivo SEB-stimulated cells when 96 mg/kg of pentoxifylline was used. Although no inhibition of TNFα was observed at 96 mg/kg of pentoxifylline, 78% attenuation was observed at 120 mg/kg. IFNγ and IL-2 were suppressed further at 120 mg/kg of pentoxifylline. Compared to TNFα, IFNγ and IL-2 were more sensitive to inhibition by pentoxifylline. A similar degree of inhibition of the three cytokines was observed using SEA-stimulated cells at different doses of pentoxifylline.

We also compared the effects of 120 mg/kg of pentoxifylline at 3 h (after the first dose) and at 27 h (3 h after the fourth dose) with blood cells taken from sham-treated

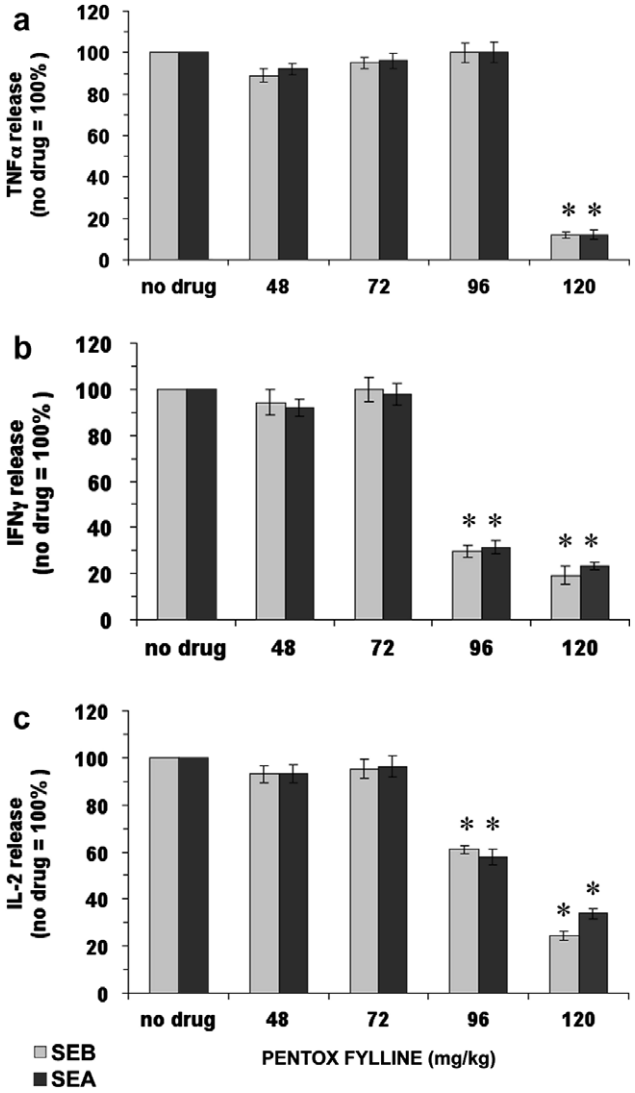

Fig. 2. Inhibition of TNFα, IFNγ, and IL-2 production by whole-blood cells stimulated by SEB or SEA after oral dosing of 0, 48, 72, 96, and 120 mg/kg of pentoxifylline at 27 h. Results are expressed as a percentage of SE-stimulated whole-blood cell cultures of NHP at zero time (untreated NHP). Data show mean inhibition ± SE. * denotes a statistical significant difference ($p < .05$) between pentoxifylline and untreated groups.

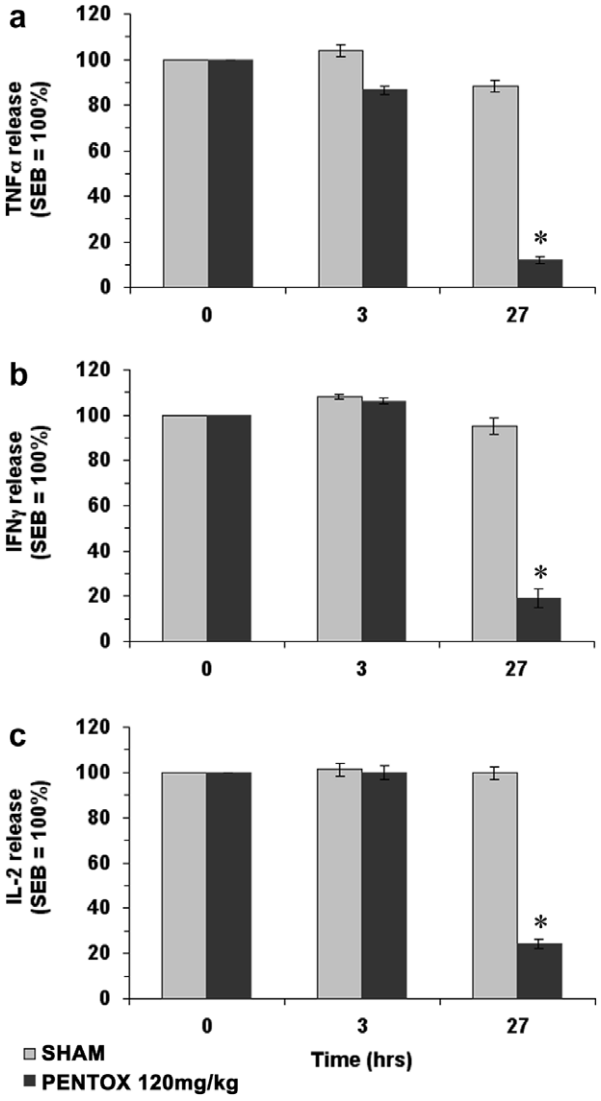

Fig. 3. Inhibition of TNFα, IFNγ, and IL-2 production by whole-blood cells stimulated by SEB after oral dosing of 120 mg/kg pentoxifylline at 3 h or 27 h. Results are expressed as a percentage of SE-stimulated whole-blood cell cultures of NHP at 0 h (untreated = 100%). Sham-treated NHP were treated identically except water was given instead of pentoxifylline. * denotes a statistical significant difference ($p < .05$) between pentoxifylline and sham-treated groups.

NHP at the same time points ($n = 4$). Cytokine levels did not change in sham-treated controls with blood cells taken at 3 or 27 h (Fig. 3). No effect of pentoxifylline was observed at 3 h, whereas inhibition of TNFα, IFNγ and IL-2 to 88%, 81% and 76%, respectively, was evident at 27 h after oral dosing of 120 mg/kg.

4. Discussion

The results presented herein indicate that SEB and SEA can induce monkey whole-blood cells to produce the typical cytokines induced by human peripheral blood mononuclear cells upon stimulation by superantigens. The use of whole-blood cells ex vivo affords the evaluation of drug

doses and effects on NHP, an animal model much more akin to human. Moreover, the biological effect(s) of a given drug is a more powerful tool in examining drug efficacy than other parameters and will aid in the discovery of drug targeting specific diseases when surrogate markers of diseases are available. For the first time, this study uses whole blood cells ex vivo to evaluate drug potency in NHP based on the biological response to a specific biotoxin.

Pentoxifylline has long been used clinically for the treatment of peripheral arterial disease (Regensteiner and Hiatt, 2002). Peak plasma levels of pentoxifylline as well as its metabolites are reached within 1 h (Physicans' Desk Reference 2005). The plasma half-life of pentoxifylline and its metabolites varies from 0.4 to 1.6 h. Thus the ex vivo anti-cytokine effects of pentoxifylline seen in this study is likely a result of its immunosuppressive effects on drug-treated cells in vivo as its plasma pharmaco kinetics indicates rapid clearance. Although the effect of pentoxifylline in microcirculation is well known, its use in modulating inflammation has recently been investigated in various in vitro and in vivo models of acute inflammation (Krakauer and Stiles, 1999; Bell, 2005; Whitehouse, 2004). In the present study, we assessed its effectiveness in targeting the immunological activating effects of a bacterial super antigen in NHP.

In summary, we evaluated the anti-inflammatory potency of pentoxifylline by oral dosing NHP and performed ex vivo measurement of cytokine release from superantigen-stimulated whole-blood cells taken at various intervals after different oral regimens. Suppression of cytokine response was not observed at lower doses of pentoxifylline (48 mg/kg, 72 mg/kg) at either 3 h or 27 h after initiation of the first dose. We found that 96 mg/kg of pentoxifylline given orally to NHP effectively inhibited SEB-induced IFNγ and IL-2 in ex vivo cultures of NHP whole-blood cells at 27 h. Thus the measurement of cytokine release from stimulated whole-blood cells of NHP after initiation of drug treatment provides a convenient and measurably functional correlate of drug potency and defines the exact dose of drug necessary to target specific responses.

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